Antimicrobial Activities of some Herb and Spices Extracted by Hydrodistillation and Supercritical Fluid Extraction on the Growth of *Escherichia coli*, *Salmonella* Typhimurium and *Staphylococcus aureus* in Microbiological Media

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Abstract

This study investigated the antimicrobial actions of *Zanthoxylum limonella*, neem leaves, garlic and galangal from Laos to inhibit some foodborne pathogens, particularly *Escherichia coli*, *Salmonella enterica* serovar. Typhimurium and *Staphylococcus aureus*. Herb extracts were obtained by hydrodistillation at 100ºC for 4 h at atmospheric pressure or by supercritical fluid extraction at 45ºC and 17 MPa for 4 h. The antimicrobial activities of the extracts were then studied against three different pathogens on microbiological media using Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and agar disc diffusion assay. The highest yield extract was determined in the *Z. limonella* extract obtained by hydrodistillation, which was 6.32±0.40%. In the MIC method, the *Z. limonella* extract from hydrodistillation and galangal extract obtained by supercritical fluid extraction at a concentration of 12.5% could inhibit all of the studied pathogens. However, it was only the *Z. limonella* extract produced by hydrodistillation that could kill the pathogens at the lowest concentration of 12.5%. Regarding the agar disc diffusion assay, *Z. limonella* extract from hydrodistillation at 100% concentration could inhibit *E. coli* for 15.67±1.81 mm, which was not significantly different to that of an antibiotic control of 10 μg methicillin ($p \geq 0.05$). For *S.* Typhimurium and *Staph. aureus*, the highest inhibition could be significantly achieved by galangal extract obtained by supercritical fluid extraction at 100% concentration for 25.87±0.68 and 25.77±0.68 mm, respectively ($p < 0.05$). These inhibition zones were significantly higher than their antibiotic controls of 10 μg amoxycillin and 10 μg methicillin, respectively.

Keywords: Antimicrobial activity, Herb extracts, Hydrodistillation, Supercritical fluid extraction

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1. Introduction

Essential oils of herbs and spices are highly concentrated, volatile, natural, complex compounds characterized by a strong odor and are formed by aromatic plants (Bakkali *et al*., 2008; Stanojević *et al*., 2015). The compounds are normally formed in special cells or groups of cells, found in leaves and stems, and commonly collected in one specific region, such as flowers, buds, seeds, leaves, bark, herbs or fruits (Gutierrez *et al*., 2008; Bajpai *et al*., 2012). They possess antimicrobial and antioxidant activities due to small terpenoids and phenolic compounds, including thymol, carvacrol, eugenol (Dušan *et al*., 2006; Seydim and Sarikus, 2006; Bajpai *et al*., 2012). Since the essential oils are recognized as natural antibacterial compounds, their application in food is an attractive way to control the presence of pathogenic bacteria and/or to extend the shelf life of processed food (Bajpai *et al*., 2012). This would also be an alternative approach to chemical additives (Shylaja and Peter, 2004) and fulfilled a demand of consumers, who preferred food products without preservative agents (Oonmetta-aree *et al*., 2006; Martínez-Graciá *et al*., 2015).

Some herb and spices, such as *Z*. *limonella*, neem leaves, garlic and galangal, were appealed to be studied. Studies of *Z*. *limonella* have been reported by some researchers (Negi *et al*., 2011; Tangjitjaroenkun *et al*., 2012a; Supabphol and Tangjitjaroenkun, 2014). These researchers conveyed that roots, leaves, fruits, stems and stem-barks of *Z*. *limonella* were utilized as spices and traditional medicines. According to Tangjitjaroenkun *et al*. (2012a), stems of *Z*. *limonella* possessed antifungal activity and contained limonelone, (-)-asarinin, dihydroalatamide, (-)-tembamide, dictamnine and *N*-nornitidine. On the other hand, limonene, (+)-sabinene and terpinen-4-ol were three major compounds found in *Z*. *limonella* oil (Charoensup *et al*., 2016). A review by Supabphol and Tangjitjaroenkun (2014) reported that essential oil of fruit *Z*. *limonella* could inhibit *Bacillus cereus*, *E*. *coli*, *Listeria monocytogenes*, *Salmonella* Rissen, *Pseudomonas fluorescens* and *Staph*. *aureus*. Neem or *Azadirachta indica* that is widely found in South Asia, Southeast Asia and West Africa, is known as a medicinal plant (Mongkholkhajornsilp *et al*., 2005; Subapriya and Nagini, 2005). Leaves of the tree were extensively used as a vegetable and neem oil contained some bitter compounds, including nimbin, nimbinin, nimbidin and nimbidiol (Mongkholkhajornsilp *et al*., 2005). Subapriya and Nagini (2005) reviewed that mahmoodin, a limonoid isolated from *A*. *indica* displayed a significant antibacterial activity. A work of Maragathavalli *et al*. (2012) demonstrated that methanol and ethanol extracts of neem leaves could inhibit *Bacillus pumilus*, *Pseudomonas aeruginosa* and *Staph*. *aureus* in an agar disc diffusion method, while the extracts could not prevent the growth of *E*. *coli* and *S*. Typhimurium.

Garlic or *Allium sativum* is part of the *Allium* genus and is widely studied spice with many purported benefits. It contains organosulphur compounds with antioxidant, anti-inflammatory and antimicrobial properties (Wilson and Demmig-Adams, 2007). The spice has an active substance allicin (diallyl thiosulfate) that is responsible for the pungent smell and for its therapeutic properties (Santhosha *et al*., 2013). Ankri and Mirelman (1999) reviewed that different garlic preparations exhibited a wide spectrum of antibacterial activity against Gram negative and Gram positive bacteria, including species of *Escherichia*, *Salmonella*, *Staphylococcus*, *Streptococcus*, *Klebsiella*, *Proteus*, *Bacillus*, *Clostridium* and *Mycobacterium*. Beside garlic, galangal (*Alpinia galanga*) in the family Zingiberaceae is widely cultivated in Southeast Asia countries, such as Philippines, Indonesia, Thailand, India and China. The herb is commonly used in diets as well as in traditional medicines (Wu *et al*., 2014). It is rich in phenolic compounds, viz. flavonoids and phenolic acids (Mayachiew and Devahastin, 2008). A study by Oonmetta-aree *et al*. (2006) reported that a major constituent of galangal ethanol extract was D,L-1'-acetoxychavicol acetate. At a concentration of 1000 µg/mL of galangal essential oils, it could inhibit *Staph*. *aureus*, *S*. Typhimurium and *Vibrio cholerae* (Hamad *et al*., 2016).

Foodborne diseases can produce negative public health outcomes, causing morbidity and mortality. There have been three major pathogens that constantly be studied since 1990's, which were *Salmonella*, *Campylobacter* spp. and *E*. *coli* (Das *et al*., 2017). *E*. *coli* cells are characterized as Gram negative, chemoorganothrophic and oxidase negative commensal microorganisms. Pathogenic *E*. *coli* have been recognized as the leading cause of traveller's diarrhea (40–70% reported cases) (Baker *et al*., 2016). The pathogen presents in human intestines and causes urinary tract infection and coleocystitis or septicaemia (Zhang *et al*., 2016). *Salmonella* is the most commonly reported source of food poisoning around the world. The bacteria are Gram negative, facultatively anaerobic and non-spore forming bacilli and have been identified for more than 2000 serotypes (Fadil *et al*., 2018). *S*. Typhimurium is an important foodborne pathogen in all regions in the world and it can cause different diseases, ranging from self-limiting gastroenteritis to systemic infection, and to infect many different hosts, including humans (Herrero-Fresno and Olsen, 2018). *S*. *aureus* is Gram positive cocci, which is belong to staphylococci genus that has 33 species. Most staphylococci constitute the normal flora of the skin and mucus membrane (Almasaudi *et al*., 2017). The microorganism is mostly accountable for food poisoning, toxic shock syndrome, endocarditis and post-operative wound infections (Zhang *et al*., 2016).

The objective of this study was to compare the antimicrobial activities of *Z*. *limonella*, neem leaves, garlic and galangal extracted by either hydrodistillation or supercritical fluid extraction method against three major pathogens of *E*. *coli*, *S*. Typhimurium and *S*. *aureus* in microbiological media.

2. Materials and Methods

2.1 Raw materials of herb and spices

Dried herb and spices of *Z*. *limonella*, neem leaves, garlic and galangal were purchased from morning markets in Vientiane capital city, Laos, sliced into pieces for garlic and galangal (approximately 0.1 to 0.2 mm in thickness), and dried using sun drying for about 7 days. The dried herb and spices were then transferred to the Faculty of Agro-Industry, Chiang Mai University, Thailand for approximately 14–16 h at room temperature. In Chiang Mai University, the dried raw materials were kept at 4ºC. On the day of extraction, each raw material was ground using a laboratory blender (Philips blender, Indonesia) and sieved through 1 mm pore size mesh (Wongsrisom *et al*., 2014; Lins, 2018). All of the dried herb and spices were measured for their moisture content, water activity and color values (Rachkeeree *et al*., 2014).

2.2 Source and maintenance of bacterial pathogens

Three bacterial pathogens, including *E*. *coli* TISTR 073, *S*. Typhimurium TISTR 2519 and *Staph*. *aureus* TISTR 2329, were obtained from Thailand Institute of Scientific and Technological Research (TISTR), Pathum Thani, Thailand. The pathogens that came as freeze-dried cultures were revived according to the TISTR guidelines using Tryptone Soya Broth (TSB; Himedia, India). The resuscitated cultures were aerobically cultivated in TSB medium at 37±1ºC for 24 h (Seydim and Sarikus, 2006), mixed with sterile glycerol (Merck, Germany) at ratio 1:1 and stored at -20ºC as stock cultures. When the pathogens were used in an experiment, the bacteria were activated by transferring 0.05 mL of stock cultures into 10 ml sterile fresh TSB and incubated at 37±1ºC for 24 h (Samelis *et al*., 2003).

2.3 Hydrodistillation extraction

Each herb or spice studied in this research was extracted by a hydrodistillation method using a Clevenger type apparatus. In each extraction process, herb or spice powder was mixed with distilled water at a ratio of 1:10 (w/v) in a round bottom flask on a heater (Samadi *et al*., 2017). To prevent spout development, glass balls were also added into the flask. The extraction process was carried out at a heating power of 90–100ºC for 4 h at atmospheric pressure. At the end of the extraction process, the obtained extract was dried over anhydrous sodium sulfate (Fadil *et al*., 2018) and kept in a dark sealed bottle at 4ºC until

used in experiments (Mejri *et al.,* 2010). This extract was recognized as 100% concentration solution. The yield of each herb extract was calculated based on Equation.

Yield of extract (%) = (Volume of the extract) / (Weight of sample) × 100

2.4 Supercritical fluid extraction

Supercritical carbon dioxide extraction was used as another method to extract the studied herb and spices because most soluble components, such as essential oils, could be extracted by the technique (Reverchon and De Marco, 2006). The extraction process was carried out using 1,000 g of ground samples in an extractor vessel. The dried herb and spices were extracted at a pressure of 17 MPa and a temperature of 45ºC (Salea *et al*., 2017) for 3 h extraction time (Zhao and Zhang, 2014) using carbon dioxide gas at a flow rate of 46.1 to 143.9 L/h. The collected extract solution was recognized as 100% concentration, stored at 4ºC prior to analyses in order to prevent chemical reactions, such as isomerization and oxidation (Bagheri *et al*., 2014) and calculated for its yield based.

2.5 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of herb and spice extracts

Prior to MIC and MBC experiments, pathogen cultures were cultivated in sterile TSB at 37±1ºC for 24 h. After the incubation period, the cultures were serially diluted in 0.1% peptone water (Himedia, India) and enumerated using Tryptone Soya Agar (TSA; Himedia, India). Plates of the pathogen cultures were aerobically incubated at 37±1ºC for 48 h. After the incubation period, it was found that *E. coli* in the solution was 1.00×10^{9} cfu/mL, *S*. Typhimurium was 2.88×10^{8} cfu/mL and *Staph. aureus* was 1.35×10^{9} cfu/mL. For the MIC and MBC studies, these cultures were diluted in 0.1% peptone water to obtain approximately 10^{7} cfu/mL.

MIC and MBC determination were carried out in a sterile 96 well plate. Into each of the well, 125 μL of sterile TSB, 15 μL of bacteria cultures of either *E. coli*, *S*. Typhimurium or *Staph. aureus*, 0.5 μL of sterile Tween 80 and 10 μL of herb extract or sterile distilled water were added and mixed properly. The concentrations of herb extract investigated in this experiment were 100.0, 50.0, 25.0 and 12.5%. Sterile distilled water was used as a negative control (no addition of herb extract). For positive controls, the wells were added with an antibiotic disc, 10 μL sterile distilled water instead of herb extracts and 0.5 μL of Tween 80 was replaced with 0.5 μL of sterile distilled water. The positive controls included 10 μg amoxycillin (Oxoid, UK), 10 unit methicillin (Oxoid, UK) and 10 μg penicillin G (Oxoid, UK). The plate was then aerobically incubated at 37±1ºC for 24 h (Turgis *et al*., 2012). The observation of the wells after 24 h incubation time was recorded as MIC. The MIC was defined as the lowest concentration of herb or spice extract that could inhibit the growth of

microorganisms in the well after 24 h incubation (the well was not turbid or had clear solution) (Turgis *et al*., 2012; Rachkeeree *et al*., 2014; Mahlangu *et al*., 2017). For MBC, solutions from the wells that were not turbid were further streaked on solidified TSA medium and incubated at 37±1ºC for 24 h. The lowest concentration of herb or spice extract that could inhibit the bacterial growth on TSA was noted as MBC (Rachkeeree *et al*., 2014; Zhang *et al*., 2016). Each test was performed in triplicate.

2.6 Agar disc diffusion assay of herb and spice extracts

The agar disc diffusion assay was carried out using a bacterial population of 10^6 cfu/mL (Shan *et al*., 2007). In brief, the studied pathogens were cultured in TSB at 37±1ºC for 24 h, diluted with 0.1 peptone water to reach the intended bacterial population, spread on solidified TSA medium using 0.1 mL of the diluted solution and left for 5–10 min at room temperature. An amount of 10 μL herb or spice extracts was dropped on a filter paper disc (6 mm) in a laminar flow cabinet. The studied concentrations of the extracts were 100.0, 50.0, 25.0 and 12.5%. After a while, the disc containing the extract was aseptically placed on the inoculated TSA plates and incubated at 37±1ºC for 24 h (Zhang *et al*., 2016; Gutiérrez-Morales *et al*., 2017). For positive controls, antibiotic discs of 10 μg amoxycillin, 10 unit methicillin and 10 μg penicillin G were used, while negative control was done by adding 10 μL sterile distilled water into a paper disc. The results were recorded by measuring the zone growth inhibition (clear zone) around the discs at 6 different positions using a digital caliper. The antibacterial activity was reported in diameter inhibition zone (mm) and the experiments were conducted in triplicate (Gupta *et al*., 2015).

2.7 Statistical analysis

Collected data were statistically analyzed in Factorial in Completely Randomized Design using SPSS version 17.0 software (SPSS Inc, Chicago, IL, USA). Duncan's multiple range tests were used to assess the difference between treatment means. A probability level $p<0.05$ was used as a statistical significance of the sample treatments.

3. Results and Discussion

3.1 Physicochemical properties of dried ground herbs and yield of herb extracts

Physicochemical properties of 4 dried herb and spices studied in this research can be seen in Table 1. The moisture content of all of the raw materials was in the range of 8.76±0.27 to 18.58±0.89%. The highest moisture content value was determined in *Z. limonella*. The moisture content of galangal powder was closed to the previous report of Rachkeeree *et al*. (2014). For the moisture content of neem leaves, it was much lower than the moisture content of the fresh material, which was reported to be 59.49 g/100g (Subapriya

and Nagini, 2005). The low moisture contents of these raw materials could assist in reducing degradation of antioxidant compounds by enzyme activities (Suhaj, 2006). The water activities of 4 herb and spices used in this study was between 0.68±0.02 and 0.73±0.00. These values were slightly higher than water activities of lemongrass, clove, garlic, ginger and cinnamon studied by Rachkeeree *et al*. (2014). Differences in the water activity values could be affected by the types of herb and spice and drying conditions given to commodities. Dried herb and spices used in this experiment had dark color with L* value of 47.72±0.41 to 61.66±0.42 with slight red color direction (positive a* value), except neem leaves, and yellow color direction (positive b* value). The results of color measurement indicated that each herb and spices had their own particular color values.

Yields of different herb and spice extracts produced by hydrodistillation or supercritical fluid extractions are presented in Table 2. The yield results displayed that the extraction method affected the quantity of the final extract. For *Z*. *limonella* extract, it was better to be processed by the hydrodistillation technique, which significantly produced the highest yield of 6.32±0.40% (p<0.05). On the other hand, neem leaves, garlic and galangal were better to be extracted by supercritical fluid extraction to produce higher yields. The result of *Z*. *limonella* extract by hydrodistillation in this study was higher than the yield of *Zanthoxylum xanthoxyloides*, which was 3.88%, reported by Negi *et al*. (2011) and the yield of dried *Z*. *rhetsa* fruit of 4.92% conveyed by Bubpawan *et al*. (2015). However, this result was lower than findings of Tangjitjaroenkun *et al*. (2012c) and Supabphol and Tangjitjareonkun (2014) for *Z*. *limonella* fruit. Beside *Z*. *limonella* extract, garlic extract obtained by hydrodistillation in this study was also lower than the previous result of Rachkeeree *et al*. (2014) extracted by a similar method. Differences in these findings could be affected by variable ecological and geographical conditions, age of the plant, harvesting time and methodology of extraction (Martínez-Graciá *et al*., 2015). For the yields of four herb and spice extracts produced by supercritical fluid extraction, this study found values between 0.90±0.13 and 1.27±0.11%, which were lower than works of Bagheri *et al*. (2014) for *Piper nigrum* L., Salea *et al*. (2017) for *Zingiber officinale* var. Anarum and Zhao and Zhang (2014) for *Eucalyptus* leaves. However, Petrović *et al*. (2016) found an extraction yield of 0.58% for supercritical extract of *Thymus praecox*. It was previously noted that particle size, pressure, temperature, extraction time, solvent flow rate, percentage of co-solvents, solubility of the solute in the fluid, diffusion through the matrix and collection process affected the yields of plant extract by supercritical fluid extraction (Reverchon, 1997; Reverchon and De Marco, 2006; Pourmortazavi and Hajimirsadeghi, 2007).

Table 1 Physicochemical properties of dried ground herb and spices

Physicochemical properties		*Zanthoxylum limonella*	Neem leaves	Garlic	Galangal
Moisture content (%)		18.58±0.89[a]	14.09±0.23[b]	14.98±0.39[b]	8.76±0.27[c]
Water activity		0.68±0.02[b]	0.73±0.00[a]	0.69±0.00[b]	0.73±0.00[a]
Color	L* value	47.72±0.41[c]	48.12±0.46[c]	58.12±0.09[b]	61.66±0.42[a]
	a* value	2.92±0.08[c]	-2.53±0.17[d]	6.73±0.04[b]	7.64±0.17[a]
	b* value	6.29±0.09[c]	13.76±0.55[b]	23.20±0.02[a]	22.29±0.77[a]

Note: [a-d] Means followed by different letters within the same row were significantly different (p<0.05).

Table 2 Yields of different herb extracts processed by hydrodistillation and supercritical fluid extraction

Herbs	Hydrodistillation (%)	Supercritical fluid extraction (%)
Zanthoxylum limonella	6.32±0.40[a]	0.90±0.13[b]
Neem leaves	0.11±0.02[c]	1.15±0.06[b]
Garlic	0.14±0.04[c]	1.27±0.11[b]
Galangal	0.11±0.01[c]	1.15±0.17[b]

Note: [a-c] Means followed by different letters were significantly different (p<0.05).

3.2 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of herb and spice extracts against bacterial pathogens

MIC values of *Z. limonella*, neem leaves, garlic and galangal extracts produced by hydrodistillation or supercritical fluid extraction against *E. coli*, *S.* Typhimurium and *Staph. aureus* are displayed in Table 3. For control treatments of the studied pathogens, it is exhibited in Table 4. From Table 3, it could be seen that extraction methods affected the antimicrobial activities of the studied herb and spice extracts. In general, the *Z. limonella*, neem leaves and garlic extracts had better antimicrobial activities when they were processed by hydrodistillation method, while lower MIC values were produced by galangal extract produced by supercritical fluid extraction compared to those of the hydrodistillation procedure. This finding could be affected by different chemical compositions in the extracts processed by different methods (Zhao and Zhang, 2014). Bagheri *et al*. (2014) reported that the *P. nigrum* L. essential oil extracted by supercritical carbon dioxide extraction and hydrodistillation possessed similar groups of compounds in different relative abundances. Different in extract composition quantities was also observed by Wenqiang *et al*. (2007).

Among four different herb and spice extracts investigated in this study, the *Z*. *limonella* extract by hydrodistillation possessed higher antimicrobial activities against *E*. *coli*, *S*. Typhimurium and *Staph*. *aureus* compared to those of neem leaves, garlic and galangal extracts produced using a similar method (Table 3). On the other hand, the galangal extract processed by supercritical fluid extraction gave a better antimicrobial activity against the studied pathogens compared to the other herb and spice extracts. The *Z*. *limonella* result in this study might be affected by the presence of limonene, (+)-sabinene and terpinen-4-ol in the herb extract as stated by Supabphol and Tangjitjareonkun (2014), Wongsrisom *et al*. (2014) and Charoensup *et al*. (2016). This finding was also parallel with the review of Supabphol and Tangjitjareonkun (2014), who conveyed that the *Z*. *limonella* fruit essential oil could inhibit several Gram positive bacteria, Gram negative bacteria and yeasts. The antimicrobial activity of galangal extract was contributed to the presence of 1,8-cineole, β-bisabolene, β-caryophyllene and β-selinene in the extract (Mayachiew and Devahastin, 2008). The authors also found that the MIC of galangal extract against *Staph*. *aureus* was 0.78 mg/mL. For Oonmetta-aree *et al*. (2006), they noted a MIC value of 0.325 mg/ml for galangal extract against *Staph*. *aureus*.

Control treatments for MIC study in Table 4 demonstrated that the three studied pathogens of *E*. *coli*, *S*. Typhimurium and *S*. *aureus* were common bacteria that could grow in microbiological broth in the absence of herb and spice extracts. At the same time, all of the bacteria were inhibited in the presence of amoxycillin and methicillin. It was interesting to note that the *E*. *coli* in this study that was isolated from human stool was resistant to penicillin. This finding was consistent to the report of Hemeg (2018). In the report, the worker informed that 120 *E*. *coli* isolates from food samples were resistance to penicillin, amoxicillin-clavulanic and erythromycin. Antibiotic resistant *E*. *coli* isolated from irrigation water and vegetables against streptomycin and tetracycline have also been previously informed by Araújo *et al*. (2017).

MBC values of different herb and spices extracts against the three studied pathogens in Table 5 showed that some of the extracts, such as garlic and galangal extracts, needed similar or higher concentration levels to kill the studied pathogens. This pattern was consistent to previous reports of Rachkeeree *et al*. (2014), Zhang *et al*. (2016) and Lahmar *et al*. (2017). Interestingly, the *Z*. *limonella* hydrodistillation extract was able to kill *E*. coli, *S*. Typhimurium and *S*. *aureus* at a concentration level of 12.5% (Table 5), which was similar to its MIC value (Table 3). A previous report of Tangjitjaroenkun *et al*. (2012b) found that essential oils of *Z*. *limonella* fruits contained 42.7% sabinene and 39.1% limonene. They also showed that the MBC of crude essential oil of the *Z*. *limonella* fruit and pure sabinene against 4 bacterial pathogens, including multi-drug resistant *S*. *aureus*, methicillin resistant *S*. *aureus*, *E*. *coli* and

extended spectrum β-lactamase producing *E. coli*, were 2.0 and 16.88 g/L, respectively. For the supercritical fluid extraction extracts, it was only the galangal extract that could kill *S.* Typhimurium at the lowest studied concentration of 12.5%. The presence of cineole (45.199%) and 4-allylphenyl acetate (13.718%) in galangal essential oil (Hamad *et al.*, 2016) might contribute to the result in this study. According to Oonmetta-aree *et al.* (2006), the ethanol extract of galangal could disrupt the cytoplasmic membrane properties and coagulate cell contents of *Staph. aureus*.

Between different herb and spice extracts, the neem leaves extract, irrespectively to the extraction methods, exhibited the lowest antimicrobial activities to inhibit and/or kill the studied pathogens. This particular extract required 100% or higher concentration to inhibit *E. coli*, *S.* Typhimurium and *S. aureus*. In the review of Subapriya and Nagini (2005), they informed that neem leaves had antibacterial activities against *Mycobacterium tuberculosis*, *Vibrio cholerae* and *Klebsiella pneumoniae*. Quelemes *et al.* (2015) also reported that ethanolic extract of neem extract was able to inhibit methicillin-resistant *S. aureus* biofilm and planktonic aggregation formation. Differences in finding could be affected by extraction methods of plant material, microorganism species, the volume of inoculum, growth phase and culture medium that was used (Tajkarimi *et al.*, 2010).

The control treatments for MBC experiment can be seen in Table 6. The results were consistent with the MIC control treatments (Table 4) that all of the studied pathogens in this study could be killed by antibiotics of amoxycillin and methicillin. For penicillin, it was bactericidal against *S.* Typhimurium and *S. aureus*, but it could not kill *E. coli*.

Table 3 Minimum Inhibitory Concentrations (%) of herb and spice extracts against the studied pathogens

Studied pathogens	Herb extracts	Minimum Inhibitory Concentrations (%)	
		Hydrodistillation extraction	Supercritical fluid extraction
Escherichia coli	*Zanthoxylum limonella*	12.5	50.0
	Neem leaves	100.0	>100.0
	Garlic	50.0	100.0
	Galangal	25.0	12.5
Salmonella Typhimurium	*Zanthoxylum limonella*	12.5	100.0
	Neem leaves	100.0	100.0
	Garlic	50.0	100.0
	Galangal	50.0	12.5
Staphylococcus aureus	*Zanthoxylum limonella*	12.5	100.0
	Neem leaves	100.0	100.0
	Garlic	50.0	50.0
	Galangal	25.0	12.5

Table 4 Minimum Inhibitory Concentration of control treatments against the studied pathogens

Studied pathogens	Sterile distilled water	Amoxycillin 10 µg	Methicillin 10 µg	Penicillin G 10 unit
Escherichia coli	+*	-	-	+
Salmonella Typhimurium	+	-	-	-
Staphylococcus aureus	+	-	-	-

Note: * (+) = cloudy well (microorganism growth) and (-) = well was not cloudy (microorganism did not growth)

Table 5 Minimum Bactericidal Concentrations (%) of herb and spice extracts against the studied pathogens

Studied pathogens	Herb extracts	Minimum Bactericidal Concentrations (%)	
		Hydrodistillation extraction	Supercritical fluid extraction
Escherichia coli	*Zanthoxylum limonella*	12.5	50.0
	Neem leaves	100.0	>100.0
	Garlic	100.0	>100.0
	Galangal	100.0	50.0
Salmonella Typhimurium	*Zanthoxylum limonella*	12.5	100.0
	Neem leaves	>100.0	>100.0
	Garlic	>100.0	100.0
	Galangal	50.0	12.5
Staphylococcus aureus	*Zanthoxylum limonella*	12.5	50.0
	Neem leaves	100.0	100.0
	Garlic	50.0	100.0
	Galangal	100.0	100.0

Table 6 Minimum Bactericidal Concentration of control treatments against the studied pathogens

Studied pathogens	Sterile distilled water	Amoxycillin 10 µg	Methicillin 10 µg	Penicillin G 10 unit
Escherichia coli	+*	-	-	+
Salmonella Typhimurium	+	-	-	-
Staphylococcus aureus	+	-	-	-

Note: * (+) = cloudy well (microorganism growth) and (-) = well was not cloudy (microorganism did not growth)

3.3 Antimicrobial activities of herb and spice extracts by agar disc diffusion method

The antibacterial activity results of herb and spice extracts against some Gram negative and Gram positive bacteria assessed by agar disc diffusion method are presented in Tables 7–9. In general, it could be seen that the antimicrobial activities of herb and spice extracts against the pathogens were decreased with lower concentration of the extracts. The *Z. limonella* and galangal extracts had higher antimicrobial activities compared to those of neem leaves and garlic extracts. The highest inhibition zone for the growth of *E. coli* was exhibited by the *Z. limonella* hydrodistillation extract, followed by garlic hydrodistillation extract, galangal supercritical fluid extract and *Z. limonella* supercritical fluid extract (Table 7). The inhibition zones of these extracts at 100% concentration were not significantly different to that of the antibiotic control methicillin, which was 17.71±0.62 mm ($p \geq 0.05$). Previously, Oonmetta-aree *et al*. (2006) had interpreted the susceptibility of a bacterium towards herb and spice extracts based on its diameter inhibition zone. The workers wrote down that a microorganism would be resistant if the diameter inhibition zone was ≤ 9 mm, intermediate if the diameter inhibition zone was between 10 and 13 mm and susceptible if the diameter inhibition zone was ≥ 14 mm. Based on these categories, it could be determined that *E. coli* was sensitive towards *Z. limonella* extract, garlic hydrodistillation extract and galangal supercritical fluid extract. Finding in this study was higher than the report of Wannissorn *et al*. (2005), who found that the diameter inhibition zones of *A. galanga* and *Z. limonella* against *E. coli* were 10.5 and 13.5 mm, respectively. On the other hand Oonmetta-aree *et al*. (2006) found that the ethanol extract of galangal could not inhibit *E. coli*. Differences in the finding could be affected by different conditions during herb extractions and microbial strains (Wannissorn *et al*., 2005; Tajkarimi *et al*., 2010). The effectiveness of garlic hydrodistillation extract against *E. coli* could be contributed to the presence of allicin, an oxygenated sulfur compound, which could inhibit certain thiol-containing enzymes in microorganisms (Ankri and Mirelman, 1999).

In the case of *S*. Typhimurium, the galangal supercritical fluid extract and *Z. limonella* supercritical fluid extract at their highest concentrations significantly had higher diameter inhibition zones compared to those of the antibiotic controls amoxycillin, methicillin and penicillin ($p<0.05$; Table 8). Based on microbial susceptibility grouping, *S*. Typhimurium was also sensitive towards galangal and *Z. limonella* supercritical fluid extracts. This result was consistent to the previous report of Wannissorn *et al*. (2005). The workers found that *A. galanga* and *Z. limonella* could inhibit *S*. Typhimurium for 15.0 and 20.5 mm, respectively. On the other hand, Weerakkody et al. (2010) found that water, ethanol and hexane extracts of *A. galanga* could only inhibit *S*. Typhimurium for 5.5, 5.5 and 6.0 mm, respectively.

For *Staph*. *aureus*, the highest inhibition zone of the pathogen growth was presented by galangal supercritical fluid extract at 100% concentration, which was also significantly higher than those of the antibiotic controls ($p<0.05$; Table 9). The *Z*. *limonella* extract at 100% concentration, irrespectively to the extraction methods, could also inhibit the last bacteria and had the antimicrobial capability that was not significantly differed than that of the methicillin ($p\geq0.05$). The sensitivity of *Staph*. *aureus* towards galangal and *Z*. *limonella* extracts found in this study was parallel with the finding of Weerakkody *et al*. (2010) for ethanol and hexane extracts of *A*. *galanga*, the review of Supabphol and Tangjitjareonkun (2014) for essential oil of *Z*. *limonella* fruit and the work of Tangjitjaroenkun *et al*. (2012b) for crude essential oil of *Z*. *limonella* fruit and sabinene. The sensitivity of Gram positive bacteria, such as *Staph*. *aureus*, towards herb and spice extracts could be due to the capability of antibacterial substances to penetrate through the bacterial cell wall and attack the cytoplasmic membrane, leading to leakage of the cytoplasm and/or cytoplasm coagulation (Weerakkody *et al*., 2010)

4. Conclusion

Based on the assessment in microbiological medium, it could be concluded that *Z*. *limonella* and galangal extract had bacteriostatic and bactericidal activities against the three studied pathogens, including *E*. *coli*, *S*. Typhimurium and *S*. *aureus*. The antimicrobial capabilities of the extracts were significantly affected by their concentration level. In addition, the extraction methods applied to the herb and spices played an important role for the extract antimicrobial actions. A further study of *Z*. *limonella* and galangal extracts in food commodity would be needed to confirm their antimicrobial activities in a real food system.

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Table 7 Diameter inhibition zones (mm) of different herb extracts against *Escherichia coli* in the agar disc diffusion method

		Herb extracts/controls							
Extraction method	**Concentration (%)**	***Zanthoxylum limonella***	**Neem leaves**	**Garlic**	**Galangal**	**Sterile distilled water**	**Amoxycillin 10 µg**	**Methicillin 10 µg**	**Penicillin G 10 unit**
Hydro-distillation	100.00	15.67±1.81^{ab}	11.96±1.11^{bcde}	14.86±0.37^{abc}	9.72±0.73^{defgh}				
	50.00	11.31±1.59^{cdef}	6.00±0.00^{h}	13.62±0.16^{abc}	7.53±0.39^{fgh}				
	25.00	10.74±1.61^{cdefg}	6.00±0.00^{h}	12.39±0.02^{bcd}	6.37±0.64^{h}				
	12.50	8.63±0.57^{efgh}	6.00±0.00^{h}	6.00±0.00^{h}	6.00±0.00^{h}				
Super-critical fluid extraction	100.00	14.22±1.74^{abc}	6.00±0.00^{h}	10.85±0.74^{cdef}	14.33±0.59^{abc}				
	50.00	7.00±0.76^{gh}	6.00±0.00^{h}	8.30±0.07^{efgh}	11.89±0.25^{bcde}				
	25.00	6.00±0.00^{h}	6.00±0.00^{h}	6.00±0.00^{h}	9.30±0.26^{efg}				
	12.50	6.00±0.00^{h}	6.00±0.00^{h}	6.00±0.00^{h}	8.44±0.05^{fgh}				
						6.00±0.00^{h}	14.90±0.18^{abc}	17.71±0.62^{a}	12.39±0.92^{bcde}

Note: $^{a-h}$ Means followed by different letters were significantly different (p<0.05).

Table 8 Diameter inhibition zones (mm) of different herb extracts against Salmonella Typhimurium in the agar disc diffusion method

		Herb extracts/controls							
Extraction method	**Concentration (%)**	***Zanthoxylum limonella***	**Neem leaves**	**Garlic**	**Galangal**	**Sterile distilled water**	**Amoxycillin 10 µg**	**Methicillin 10 µg**	**Penicillin G 10 unit**
Hydro-distillation	100.00	16.55±0.50^{de}	11.98±2.17^{hi}	8.61±0.70^{jk}	14.08±0.14^{efg}				
	50.00	13.51±1.98^{fgh}	8.29±0.94^{jkl}	6.79±0.69^{kl}	11.10±0.00^{hi}				
	25.00	10.10±1.58^{ij}	7.17±1.07^{kl}	6.00±0.00^{l}	6.00±0.00^{l}				
	12.50	9.12±1.52^{jk}	6.09±0.09^{l}	6.00±0.00^{l}	6.00±0.00^{l}				
Super-critical fluid extraction	100.00	19.06±0.34^{c}	6.00±0.00^{l}	6.00±0.00^{l}	25.87±0.68^{a}				
	50.00	14.73±0.14^{efg}	6.00±0.00^{l}	6.00±0.00^{l}	22.12±1.44^{b}				
	25.00	12.42±0.67^{ghi}	6.00±0.00^{l}	6.00±0.00^{l}	17.94±0.19^{cd}				
	12.50	6.00±0.00^{l}	6.00±0.00^{l}	6.00±0.00^{l}	16.95±0.22^{cde}				
						6.00±0.00^{l}	15.91±0.50^{def}	15.04±0.63^{ef}	9.20±0.22^{jk}

Note: $^{a-l}$ Means followed by different letters were significantly different (p<0.05).

Table 9 Diameter inhibition zones (mm) of different herb extracts against *Staphylococcus aureus* in the agar disc diffusion method

		Herb extracts/controls							
Extraction method	**Concentration (%)**	***Zanthoxylum limonella***	**Neem leaves**	**Garlic**	**Galangal**	**Sterile distilled water**	**Amoxycillin 10 µg**	**Methicillin 10 µg**	**Penicillin G 10 unit**
Hydro-distillation	100.00	21.16±0.98^{bcd}	11.74±2.09^{ghi}	12.83±2.15^{ghi}	13.50±1.17^{fgh}				
	50.00	17.88±1.27^{de}	6.20±0.06^{lm}	10.21±0.18^{hijk}	11.74±0.61^{ghi}				
	25.00	10.91±3.29^{hij}	6.04±0.06^{m}	7.29±0.21^{klm}	9.49±0.32^{ijkl}				
	12.50	7.68±1.26^{jklm}	6.03±0.05^{m}	6.00±0.00^{m}	7.46±0.25^{klm}				
Super-critical fluid extraction	100.00	20.96±0.72^{bcd}	6.00±0.00^{m}	8.56±1.02^{jkl}	25.77±0.68^{a}				
	50.00	18.75±0.43^{cde}	6.00±0.00^{m}	6.00±0.00^{m}	22.76±1.85^{ab}				
	25.00	16.89±1.91^{ef}	6.00±0.00^{m}	6.00±0.00^{m}	21.34±1.06^{bc}				
	12.50	11.13±0.79^{ghi}	6.00±0.00^{m}	6.00±0.00^{m}	12.80±0.64^{ghi}				
	100.00					6.00±0.00^{m}	4.33±0.23^{f}	10.04±0.64^{bcd}	10.65±1.41^{hijk}

Note: $^{a-l}$ Means followed by different letters were significantly different (p<0.05).

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